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Monogalactopyranosides of fluorescein and fluorescein methyl ester: synthesis, enzymatic hydrolysis by biotnylated β-galactosidase, and determination of translational diffusion coefficient

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ABSTRACT

Fluorescein monoglycosides (D-galactopyranoside (FMG) and D-glucopyranoside) and their methyl ester (MFMG) have been prepared from acetobromoglucose/galactose and fluorescein methyl ester in good yields. Enzymatic hydrolysis experiments (using biotinylated **β**-galactosidase) of the *galacto* derivatives have been performed and kinetic parameters were calculated. A 15–20 times increase of the fluorescence intensity has been observed during the hydrolysis. A linear increase of fluorescence has been noted at short time and low concentration of substrate, making these compounds useful and sensitive probes for galactosidases. The magnitude of the Michaelis–Menten constant (K_m) value for MFMG is higher than that of FMG suggesting a possible conformational change of the fluorogenic substrate. K_m value for biotinylated β-Gal with FMG is lower than that for the native enzyme. This observation indicates higher substrate affinity of the biotinylated enzyme in comparison to the native enzyme. Translational diffusion coefficients have been measured, for both fluorogenic substrates and both the products, employing fluorescence correlation spectroscopy. Translational diffusion coefficients for fluorogenic substrates and the enzymatic hydrolysis products have been measured to be similar, in the range of 3.5–4.5 $\times 10^{-10}$ m^2 s^{-1}. Thus an enhancement or retardation of the enzymatic kinetics due to difference in translational mobility of substrate and product is not that apparent.

1. Introduction

Fluorogenic glycosides are important probes for reporting glycosidase activities. In such compounds, a sugar blocks a chemical group necessary for fluorescence through a glycosidic linkage. Fluorescein is a suitable dye for that purpose and fulfills two requirements: (a) high fluorescence quantum yield; (b) a free phenol function that is necessary for fluorescence and can be blocked through an enzymatically labile glycosidic linkage. In this respect, fluorescein digalactoside (FDG) is a commercially available compound for galactosidase assays (Scheme 1). However, the fluorescence production from FDG needs two enzymatic steps: first step involves hydrolysis of FDG to give fluorescein mono galactoside (FMG)—a dark (or weakly-fluorescent) compound- and the second step to give fluorescein, the final fluorescent product. For *Escherichia coli* galactosidase, the first step is 12 times slower than the second: (k_a = 1.9 μmol min^{-1} mg^{-1}; k_b = 22.7 μmol min^{-1} mg^{-1})(Scheme 1).[1]

So, the rate of fluorescence increase from FDG was found to be very slow and not correlated with the β-galactosidase activity as a linear function at short time and low concentration (<<K_m) (Scheme 1). FMG will therefore be a far better fluorogenic compound than FDG for kinetic measurements.

In spite of more and more enzymatic assay studies, the structure–function relationship for enzymes at the single enzyme level is still not understood completely.[2–4] The major hurdle in this direction is the non availability of suitable fluorogenic probe having negligible emission and significantly higher signal to noise ratio of the highly fluorescent enzymatic hydrolysis product. Moreover, photostability of the fluorescent product is also a matter of concern.

We were interested in the enzymatic activity of a single immobilized enzyme, specially β-galactosidase, at the single molecule level, using a fluorogenic galactosyl reporter. A two step hydrolysis (of FDG) leading to the final fluorescent product dramatically limits the performance of this kind of experiment as the first hydrolysis product will diffuse out of the illuminating volume of the microscope. A single step hydrolysis reaction is needed and FMG could possibly be a suitable substrate for that purpose. Commercially

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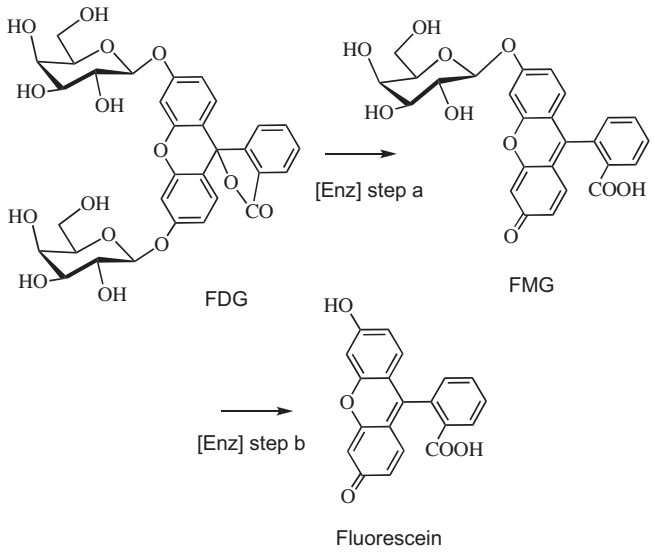

Scheme 1. Hydrolysis of FDG by galactosidase.

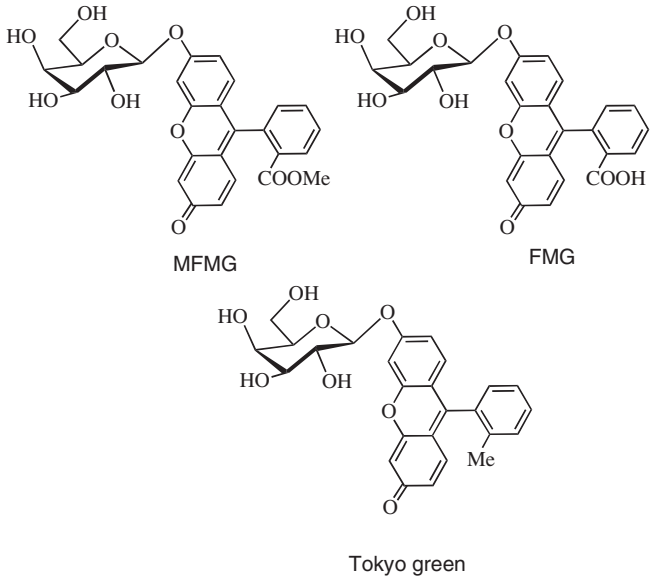

Figure 2. Chemical structures of MFMG, FMG and Tokyo green.

available alternative probes to FMG, resorufin-β-D-galactopyranoside, methylumbelliferyl-galactosides, and DDAO galactoside (9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside) have critical drawbacks.[5,6] Due to widely separated absorption profile of fluorogenic substrate and the enzymatic hydrolysis product two lasers need to be used in order to excite them. Either it is necessary to excite them in the UV region or significant overlap of the excitation and emission spectra of the enzymatic hydrolysis product reduces the sensitivity significantly.[5,6] Moreover, these dyes are not as bright as fluorescein.

In this manuscript we report detailed synthesis of Fluorescein monoglycosides (D-galactopyranoside and D-glucopyranoside) (FMG) and their methyl ester (MFMG). Characterization of all products has been made. We have employed biotinylated β-galactosidase instead of native enzyme as the former would be used for single molecule experiments. Michaelis–Menten constant (K_m) value for FMG and MFMG has been measured in order to verify whether there is any change because of chemical modification of the substrate. It is well-known that two steps of enzyme catalyzed reactions viz. (a) reversible formation of enzyme–substrate complex (ES) and (b) irreversible conversion of ES to product are determined partly by the diffusion constants of substrates. Thus we have measured the translational diffusion coefficients of both fluorogenic substrates and the products employing fluorescence correlation spectroscopy.

2. Results and discussion

2.1. Synthesis of the probes

We want to describe here the synthesis of fluorescein mono galactoside (FMG) and of their methyl ester (MFMG) and the kinetics of their enzymatic hydrolysis (Fig. 1). Strangely enough, FMG is not commercially available and no convenient synthesis of this compound is described in literature. Alternatively, Urano et al.[7] developed Tokyo green(s) (Fig. 2), mono galactosylated closely related compounds, prepared in several steps from a xanthone derivative.[8] They had shown that the presence of –COOH group on the pendant benzenic ring causes no change in the fluorescence

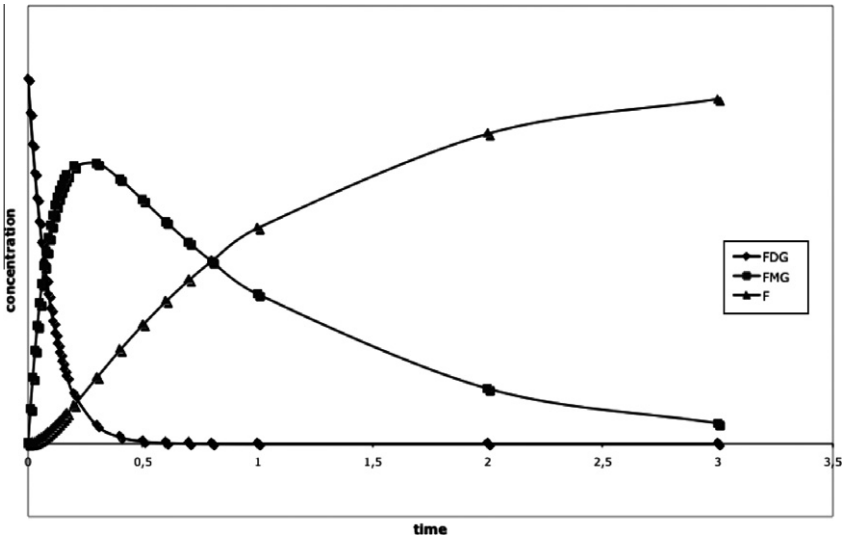

Figure 1. Kinetic profile of FDG → FMG → F sequence.